Brain Oxidative Stress in Animal Models of Accelerated Aging and the Age-related Neurodegenerative Disorders, Alzheimer’s Disease and Huntington’s Disease

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Abstract: Oxidative stress in brain is emerging as a potential causal factor in aging and age-related neurodegenerative disorders. Brain tissue from living patients is difficult to acquire; hence, animal models of aging and age-related neurodegenerative disorders, though not perfect models, have provided tissue to study the role of oxidative stress in these disorders. In this review, the central role of oxidative damage in brain models of accelerated aging (progeria and Werner’s syndrome) and the age-related neurodegenerative disorders, Alzheimer’s disease and Huntington’s disease, will be presented and evaluated. To the extent that the animal models faithfully mirror their respective disorders, and based on the totality of the studies, it is apparent that oxidative stress, the excess of free radicals over the means of scavenging these harmful agents, may play critical roles in the molecular basis of accelerated aging, Alzheimer’s disease, and Huntington’s disease.

INTRODUCTION

Aging and age-related neurodegenerative disorders, among which are Alzheimer’s disease (AD) and Huntington’s disease (HD), are becoming increasingly important as the mean age of the United States’ population increases. While AD, which presents as a progressive dementing disorder, affects millions of persons worldwide [1], HD, an inherited disorder that is characterized by progressive involuntary movements (choreiform movements) accompanied by personality and mentation changes, is relatively rare, affecting about 100,000 person in the United States [2]. Not surprisingly, the brain pathology is different in these two disorders: cortical cell loss is most pronounced in AD, while striatal neurons are principally affected in HD [1,2].

A consequence of prolonged life in an aerobic environment is increased oxidative stress with age [3,4]. Oxidative stress can be defined as a condition in which the production of free radicals and the damage they cause exceed the ability to scavenge these free radicals or repair the damage [5]. If the aging that normally occurs is accelerated, as in progeria and Werner’s syndrome, oxidative damage at a much earlier age is observed [6]. Progeria, also known as Wiedman-Rautenstrauch syndrome, is characterized by accelerated aging in persons of young chronological age [7], and Werner’s syndrome, a similar condition affecting adults [8], can provide some insight into the underlying molecular mechanisms of aging.

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The molecular basis of AD is unclear, but numerous lines of genetic and biochemical evidence suggest that a 39-43 amino acid peptide, amyloid β-peptide (Aβ) that is the principal component of senile plaques in the AD brain, is central to the pathogenesis of this disorder [9]. Aβ spontaneously forms fibrillar networks in solution and is aggregated in AD brain. HD is an autosomal dominant inherited disorder that most often presents in the fourth to fifth decade of life [2]. In this latter disorder an excessively long poly CAG repeat on chromosome 4 leads to a glutamine-rich protein termed huntingtin. The role of this protein is unknown, but recent studies indicate that this protein aggregates into long fibrillar structures that are localized to the nucleus and perhaps to other parts of the cell [10].

In each case, aging, AD, and HD, brain is under oxidative stress manifested in numerous ways, but including, protein oxidation and lipid peroxidation [4,5,11-17]. As noted above, oxidative stress results when the sources of free radicals outpace the free radical scavenging systems designed to counter these harmful, reactive agents. As one ages, one’s antioxidant defenses system declines [4]. Protein oxidation is manifested by incorporation of carbonyl functionalities into the side chain of certain amino acids [4,5,18]. Thus, this index of protein oxidation is most often employed, but other methods, particularly electron paramagnetic resonance (EPR) in conjunction with a protein-specific spin label [19], is also used to investigate protein oxidation [20-28]. The methods for detecting these markers of protein oxidation are outlined below. Lipid peroxidation, initiated after hydrogen atom abstraction from unsaturated sites on lipid acyl chains, is most often detected by formation of reactive aldehyde, i.e., malondialdehyde, or alkenal, i.e., 4-hydroxy-2-trans-nonenal (HNE) products [29]. However, oxidative stress-induced stimulation of phospholipase A₂ leads to free fatty acid release [30], and
EPR, in conjunction with lipid-specific spin labels, is used to study free radical-induced loss of EPR signal intensity of these spin labels located in the lipid bilayer [30-32].

In this review, studies using these methods, and others, for measurement of oxidative stress in animal models of accelerated aging, AD, and HD are presented. This field is expanding rapidly, and while every effort was made to include all relevant articles in this review, it is conceivable that some papers were missed. This omission should not be taken as lack of importance.

**REACTION OXYGEN SPECIES (ROS) AND THEIR MAJOR BIOMARKERS IN BRAIN**

The enzymatic and chemical formation of ROS have been reviewed recently [5]. Among others, ROS include ·OH, ·O₂⁻, H₂O₂, ROO⁻, ROOH, NO⁻, ONOO⁻, HO₂⁻, O₂⁻, HOCl, O₃, etc. In brain, the major biomarkers of the damage caused by these ROS are protein carbonyls [which as noted above are an index of protein oxidation], formation of reactive aldehydes, alkenals, and phospholipase A₂-mediated lipid fatty acid release [indices of lipid peroxidation], 3-nitrotyrosine [formed by reaction of peroxynitrite with tyrosine among other means], loss of activity of oxidatively-sensitive enzymes such as glutamine synthetase (GS) and creatine kinase (CK), oxidized DNA [usually detected as 8-hydroxy-2-deoxyguanosine], excess ROS observed by fluorescence dyes, and the presence of advanced glycation end products [resulting from Amadori chemistry]. The methods of analysis of these biomarkers of brain oxidation have been reviewed recently [5,17,19,33].

In addition to these analytical indices of oxidative stress induced by ROS in brain, EPR, in conjunction with protein- or lipid-specific spin labels, has been used to assay the effects of ROS in brain [20-28,30,31,35-37]. The most commonly used protein-specific spin label used in brain membrane studies is 2,2,6,6-tetramethyl-4-maleimidopiperidin-1-oxyl (MAL-6). This spin label binds principally to protein sulfhydryl groups, and at least two environments of SH groups, characterized by different spin label mobilities, are found on proteins [19]. As shown in Fig. (1), in W sites MAL-6 is bound to SH groups located on the surface of proteins where the spin label can undergo relatively fast rotational motion, i.e., only weakly immobilized (hence, W sites). In contrast, in S sites MAL-6 is bound to SH groups located in protein crevasses, pockets, or folds, that severely restrict the rotational motion of the spin label, i.e., strongly immobilized (hence, S sites). The ratio of the peak-to-peak signal amplitudes of the M₁ = +1 low-field resonance lines (the W/S ratio) is highly sensitive to membrane protein conformation and protein-protein interactions [19]. In all oxidative stress conditions thus far examined, ranging from hydroxyl free radical-, HNE-, peroxynitrite-, or amyloid β-peptide (Aβ)-induced damage to brain membranes [20,26-28,35], to ischemia/reperfusion injury [22-25], to Alzheimer’s disease brain [21] and models of Huntington’s disease [36,37] [see below], to lipopolysaccharide-induced membrane damage [34,38], this W/S ratio is always decreased. Such a decreased W/S ratio can emanate from altered protein conformations, decreased protein segmental motion, or increased protein-protein interactions [19]. Thus, a decreased W/S ratio of MAL-6 in brain membranes is a good index that protein oxidation has occurred.
Table I  Oxidative Stress in Age-accelerated Mice Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Average Life Span (Months)</th>
<th>Phenotypic Expression</th>
<th>Mean W/S Ratio +/- SEM (# of samples)</th>
<th>P-Value Compared to SAMR1</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAMR1</td>
<td>18.9</td>
<td>Normal Aging</td>
<td>8.22 +/- 0.11 (n = 4)</td>
<td></td>
</tr>
<tr>
<td>SAMP1</td>
<td>12.5</td>
<td>Senile Amyloidosis</td>
<td>7.15 +/- 0.05 (n = 4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SAMP3</td>
<td>16.9</td>
<td>Degenerative Joint Disease</td>
<td>6.18 +/- 0.15 (n = 4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SAMP6</td>
<td>10.7</td>
<td>Senile Osteoporosis</td>
<td>6.28 +/- 0.07 (n = 4)</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>SAMP8</td>
<td>12.1</td>
<td>Deficits in Learning and Memory</td>
<td>7.34 +/- 0.03 (n = 3)</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>SAMP10</td>
<td>11.1</td>
<td>Deficits in Learning and Memory with Brain Atrophy</td>
<td>7.59 +/- 0.11 (n = 6)</td>
<td></td>
</tr>
</tbody>
</table>

In contrast to the protein spin label MAL-6 covalently attached to SH groups, nitroxide stearate (NS) spin probes are non-covalently incorporated into the lipid bilayer [19]. Depending on the location of the nitroxide moiety, one can probe different depths within the lipid bilayer, e.g., 5-NS vs. 12-NS. These probes undergo rapid anisotropic rotation about the long axis of the probe and are excellent reporters of phospholipid bilayer order and motion [fluidity] [19]. As the bilayer becomes more fluid, reorientation of the NS spin probes can occur, and similar to the effects observed in magnetic resonance in chemical exchange, the linewidth [half-width at half-height, HWHH] of the low-field line increases [19]. In contrast, if the bilayer becomes less fluid the HWHH decreases [19]. Further, since normally the only source of paramagnetism of brain membranes is the NS spin probe itself, when ROS are formed in the bilayer they react with the unpaired electron on the spin probe, thereby decreasing the EPR spectral intensity. That is, one can use the loss of signal amplitude as a marker of lipid-resident ROS formation. Such studies have been used to study the effects of lipopolysaccharide [38] and Aβ [30-32], among others.

**ANIMAL MODELS OF ACCELERATED BRAIN AGING**

The senescence-accelerated mouse (SAM) was developed using a phenotypic selection from a common genetic pool [39]. Several different strains of SAM mice, showing different behavioral characteristics (Table I) were developed. The SAMP8 strain (age-accelerated prone) has a shortened life span [about one year compared to nearly 19 months for the age-accelerated resistant (SAMR1) mouse] and loss of memory and learning skills relative to the SAMR1 mouse [40-42]. As such, this is a good model to study brain aging in accelerated senescence.

Consistent with the notion that oxidative stress is causal in brain aging, a decreased W/S ratio of MAL-6 in cortical synaptosomal membranes, an increased level of protein carbonyls, and decreased activity of GS were observed in brain tissue from SAMP8 mice compared to those parameters in SAMR1 mice [11,43]. Treatment of SAMP8 mice daily for two weeks with the spin trap N-tert-butyl-α-phenylnitrone (PBN) abrogated the oxidative damage in these age-accelerated mice [11]. This latter result may be related to the observation that the life span of these age-accelerated mice was increased by nearly 50 % by chronic administration of PBN [44]. The increased protein oxidation in SAMP8 mice also was observed by others [45,46], and lipid peroxide content was increased in SAMP8 mouse brain [47], consistent with oxidative damage in accelerated aging. The increased oxidative damage in this model of progeria and Werner’s syndrome mimicked the observed oxidative stress in these human conditions [6].

Other strains of SAM mice were also examined for evidence of oxidative stress and treatment with PBN (Table I). In each case, the W/S ratio of MAL-6 bound to synaptosomes isolated from these animals is statistically significantly lower than that of SAMR1 mice, suggesting that oxidative damage is associated with accelerated aging. Consistent with this finding, others reported that the antioxidant melatonin protected SAMP-6 mice against age-related DNA oxidation [48].

Mitochondrial dysfunction has been proposed as a cause of oxidative damage in accelerated aging [49], and increased mitochondrial DNA deletion in SAMP8 mouse brain has been reported [50].

The status of learning and memory of SAMP8 mice has been studied extensively. SAMP8 mice have early onset of memory loss [42], but few studies have been reported on membrane structural changes in these age-accelerated mice. Higher levels of malondialdehyde, an end-product of lipid peroxidation, were reported in brain from SAMP8 mice relative to SAMR1 mouse [51], consistent with the protein oxidation findings reported from our laboratory [11]. As noted above, the free radical spin trap PBN was reported to...
extend the life span of SAMP8 mice by 50 %, essentially reaching the life span of SAMR1 mice [29]. While the molecular alterations associated with progeria and Werner’s syndrome remain unknown, age-accelerated mice may be a good model for these human conditions. For example, fibroblasts obtained from both progeria and Werner’s syndrome patients have increased protein oxidation in tissue culture [6].

Hyperoxia has been used as a model of aging [4], and rodent brains that look like those of extremely elderly rodents can be achieved in 24 h by exposure to greater than 90% oxygen; as such this is a model of accelerated aging. Gerbils exposed to nearly 100 % oxygen for 24 hours exhibited significantly increased oxidative damage to cortical synaptosomal proteins, and this damage could be prevented by pretreatment with PBN or Tempol (2,2,6,6-tetramethyl-4-ol-piperidin-1-oxyl, a spin label) [52]. These and other free radical spin traps and spin labels have been used in other oxidative stress conditions to modulate oxidative damage to brain membranes [see for example 23,37,53], and their protective effect in rodent models of accelerated aging are consonant with the notion that aging is associated with oxidative stress.

Diet restriction can extend life span [54,55]. Consistent with this observation, the increased protein oxidation and loss of activity of the brain isoform of CK associated with rodent brain aging was significantly diminished in aged animals maintained on a calorically restricted diet [56-58]. However, synaptosomal membranes from such diet-restricted animals apparently are not different from synaptosomal membranes from ad-libitum animals: if Complex II were stimulated by excess succinate, the same degree of oxidative damage was observed [58-61]. This result may suggest that one means of increased oxidative damage in aging is electron (ROS) leak from mitochondria as a result of aerobic metabolism. The increased life span in diet restriction may be due in part to the decreased ROS leak from mitochondria secondary to decreased “turning of the crank” associated with decreased metabolism. However, other processes may be involved. For example, diet restricted animals have increased expression and levels of heat shock proteins that may play a protective role [62].

Future studies of brain aging may be assisted with newly developed paradigms for neuronal cultures in which neurons are kept viable for up to 60 days [63], far longer than is normally the case. Aged neurons showed evidence of increased protein oxidation measured by Western blots and by a newly-developed in-situ method for detecting protein carbonyls [63].

In addition to age-accelerated rodents, other researchers have found increased protein oxidation in various species of flies or worms, whose life spans are short [35,64,65]. Thus, it may be a universal observation that increased oxidative damage is causal of aging. If this notion is true, then either means to decrease ROS leak from mitochondria (decreased calorie intake) or increased dietary antioxidants may be beneficial to slow the progression of aging. In progeria and Werner’s syndrome, the evidence for oxidative stress suggests that high levels of systemic antioxidants should be considered as a therapeutic approach in these disorders.

OXIDATIVE STRESS IN ANIMAL MODELS OF ALZHEIMER’S DISEASE

The pathology of AD brain is characterized by the presence of senile plaques and neurofibrillary tangles and the loss of synapses [1,66]. The major constituent of senile plaques is Ab, principally in the 42- or 40-amino acid form, Ab(1-42) and Ab(1-40), respectively. Genetic mutations in the genes that code for amyloid precursor protein (APP), presenilin-1, or presenilin-2 lead to an excess deposition of Ab(1-42) and development of familial AD. This observation, coupled with the findings that APP is coded for on chromosome 21 and Down’s syndrome patients develop AD if they live sufficiently long, has led to the hypothesis that Ab is central to the neuropathology and dementia associated with AD [9]. The AD brain is under pronounced oxidative stress, manifested by protein oxidation [21,67,68], lipid peroxidation [69-73], DNA and RNA oxidation [74-78], widespread peroxynitrite-induced damage [79], advanced glycation end products [80], and altered antioxidant enzyme activity or expression [69,81-90]. As predicted from an oxidative stress disorder, vitamin E slows the progression of AD [91]. Ab, in ways that are inhibited by free radical antioxidants like vitamin E, causes brain cell protein oxidation [26,83,84,92-96], lipid peroxidation [30,34,97-103], and ROS formation [84,94-96,104,105], among other oxidative stress responses, suggesting that this peptide is a source of oxidative stress in brain. The single methionine residue of Ab(1-42) and Ab(1-40) is essential to the oxidative stress properties of this peptide [92,106-108]. Other sources of oxidative stress in AD also are likely, ranging from altered mitochondrial function, to trace metal ion imbalances or altered metal ion binding to biomolecules, to advanced glycation end products (reviewed in Markesbery, 1997 [13]).

In hippocampal cell culture, Ab addition leads to formation of HNE [102], a reactive alkenal that binds to cysteine, histidine, and lysine residues of proteins by Michael addition [5]. HNE, like Ab, can inhibit multiple transmembrane transport proteins, such as ion-motive ATPases, glucose and glutamate transporters, and G-protein-coupled signaling pathways [reviewed in Mattson et al., 1999]. HNE alters the conformation of synaptosomal membrane proteins [35], and HNE is found in abundance in AD brain [71]. Calcium ion homeostasis is lost in cultures treated with Ab [105]. Intracellular Ca\(^{2+}\) accumulation can lead to incorporation into mitochondria, which might be related to decreased energy utilization in AD brain. A provocative suggestion has emerged that suggests that dietary restriction, known to induce protective heat shock proteins [62], may be a means of slowing or preventing AD [109]. Further studies will be required to evaluate this suggestion.

In any animal model of AD, the pathology and neurochemistry ideally should be highly reminiscent of the human disease. Unfortunately, as of this writing no animal model completely mimics all aspects of AD [reviewed in van
Leuven, 2000]. Several transgenic animal models of AD are available based on mutations in human APP or presenilin-1 [110-118], or combinations of mutations in APP and PS-1 [119,120]. In addition to mutations in APP and the presenilin genes being causally linked to inherited AD, the presence of the epsilon-4 allele of apolipoprotein E is correlatively related to development of this disorder [121]. Hence, animal models containing specific alleles of apo E or knockouts of apo E have been developed [reviews:114,122].

In transgenic animals expressing mutant APP, excess Aβ(1-42) deposition is found [110], and memory deficits, initially thought to occur after 6-9 months of age, appear to occur in these animals closer to 20 months of age [123]. This latter result may not be surprising, since a model of AD should mirror its age-dependence. What is surprising in this model of AD is that neuronal death is not observed [124]. Thus, this model, like all animal models of AD, is only partially replicative of AD pathology and neurochemistry. However, consistent with an oxidative environment in AD brain, transgenic animals expressing mutant APP that deposit excess Aβ(1-42) are reported to have increased brain protein oxidation when measured in elderly animals [125,126].

Increased Aβ(1-42) production is observed in transgenic mice expressing mutant PS-1 [127]. Brain cells or cultured neurons isolated from transgenic animals expressing mutant PS-1 have increased vulnerability to oxidative insults [118,128], suggesting that these membranes may be under oxidative damage already and the oxidative insult concentration needed to cause further damage is less. Recently, direct evidence of oxidative damage in such animals was found. Using an immunochemical assay for protein carbonyls, 4-month old transgenic mice expressing mutant PS-1 had a significantly higher level of protein oxidation than did wild-type mice [129]. Consequently, in both the APP and PS-1 transgenic mice models of AD, there is evidence of oxidative damage, similar to that seen in AD brain [21]. The molecular basis of oxidative damage in AD brain remains unknown, but the clear demonstration that Aβ(1-42) and Aβ(1-40), both present in AD brain, induce neuronal oxidative stress and neurotoxicity in ways that are inhibited by free radical scavengers coupled with the apparent centrality of Aβ to the pathogenesis of AD, suggest that Aβ must be considered a potential cause of both the oxidative damage and neurodegeneration observed in AD brain. Recent evidence suggests that caspase-2 activation is necessary for Aβ-induced apoptotic neuronal death [130], and others report that activation of the nuclear transcription factor, NFk-B, may be anti-apoptotic and beneficial in this disorder [131].

Relative to wild-type mice, apo E knockout mice have increased basal levels of lipid peroxidation [132], and this is manifested as an increased lipid fluidity as assessed by the HWHH parameter using EPR spin labeling [133]. This result suggests that, normally, apo E may play a protective role against oxidative stress. However, the allele, Apo E4, that has a proclivity to be increased in late-onset AD, contains no SH groups, unlike apo E3 and apo E2. Since the AD brain is under oxidative insult, probably in part from excess Aβ, the apo E4 isoform of this lipoprotein may not provide as much antioxidant protection as the other isoforms of this lipoprotein.

Considerably more research on animal models of AD is needed. Especially promising may be investigations of doubly transgenic animals with mutations in APP, PS-1, apo E, and tau genes [134,135]. In addition, therapeutic strategies that may be helpful in AD potentially may be examined in these animal models. In addition to rodent transgenic models of AD, a worm transgenic model of Aβ(1-42) deposition has been developed [136]. In this transgenic worm, human Aβ(1-42) is deposited in the muscle walls of Caenorhabditis elegans (C.elegans) using a unc-54 promoter. The Aβ in vivo is in a beta-sheet conformation as assessed by Thioflavin T fluorescence assays. The phenotypic expression is one of paralysis: when put on a food source, transgenic worms, in contrast to vector-only worms, do not move. Our laboratory, in collaboration with Link’s laboratory, tested the hypothesis that, since Aβ induces oxidative damage in brain cells and since AD brain is under oxidative stress and has deposition of Aβ(1-42), transgenic worms that expressed human Aβ(1-42) would exhibit oxidative stress. This hypothesis was borne out: transgenic worms expressing human Aβ(1-42) have a significantly elevated protein oxidation compared to vector-only controls [92]. In addition, to test the hypothesis that, similar to the in-vitro case noted above, the methionine residue 35 of Aβ(1-42) would be important in the oxidative stress properties of this peptide in vivo, the methionine was mutated to cysteine. In this case, no protein oxidation was noted over that of the vector control, strongly supporting the notion that methionine is critical to the free radical oxidative stress properties of this peptide [15,92,106]. Consistent with the lack of oxidative damage, this transgenic animal expressing cysteine-substituted Aβ(1-42) is not paralyzed, in marked contrast to the protein oxidation and paralysis of nematodes expressing native human Aβ(1-42). This worm model of human Aβ(1-42) deposition offers the opportunity to test various hypotheses for Aβ(1-42)-induced oxidative damage and cell death, as well as various potential therapeutic strategies. Such studies are on going in our collaborative laboratories.

Other laboratories have reportedly employed C.elegans and fruit flies to investigate presenilins [see for example, 137-139], and others have reported a homologue of PS-1 in carp retina [140]. Such animal models may provide vehicles to investigate oxidative damage resulting from a mutated PS-1 gene.

OXIDATIVE STRESS IN HUNTINGTON’S DISEASE

Huntington’s disease (HD) is a progressive neurodegenerative disorder characterized by involuntary choreiform movements, personality changes, depression, loss of cognitive function, and massive loss of neurons in the striatum [2,141]. The genetic cause of HD is an autosomal dominant mutation on chromosome 4 resulting in an expanded polyglutamine region of the huntingtin protein. The role of mutant huntingtin in neuronal degeneration is, as of yet, unknown; however, impairment of energy metabolism is believed to be involved. Huntingtin protein is expressed
systematically, and the reason for the preferential vulnerability of striatal neurons is also unknown.

Oxidative damage has been suggested to play a role in the neuropathology of HD. Several studies show evidence of oxidative fragmentation of DNA in striatal neurons of HD patients [142,143]. Evidence of DNA oxidative fragmentation has also been reported in cortical neurons, and mitochondrial DNA appears to be more susceptible that nuclear DNA [16]. This observation appears to support the hypothesis that energy metabolism may play a role in HD neurodegeneration. Other indices of oxidative stress in HD are the reported increased levels of malondialdehyde, 3-nitrotyrosine, heme oxygenase-1, and lipofuscin [16]. Also, HD fibroblasts show increased vulnerability to glutamate, a vulnerability that is inhibited by antioxidants [16]. Neuroprotective strategies against basal ganglia degeneration in HD, including the use of antioxidants, have been reviewed recently [144].

**OXIDATIVE STRESS IN ANIMAL MODELS OF HUNTINGTON’S DISEASE**

**The 3-Nitropropionic Acid Model**

Animal models of HD have been utilized to investigate the mechanisms of neurodegeneration in this disease. One common type of animal model for HD has been the use of mitochondrial toxins. Of these, the most prominent toxins have been inhibitors of succinate dehydrogenase. This enzyme, complex II of the mitochondrial electron transport chain and of the citric acid cycle, converts succinate to fumarate with the concomitant reduction of a flavin adenine dinucleotide. Though this reduction itself is of insufficient free energy to fuel the synthesis of ATP, it nevertheless is important in allowing electrons from carbohydrate metabolism a pathway into the electron transport chain. The two most widely used succinate dehydrogenase inhibitors are malonate and 3-nitropropionic acid (3-NP). While malonate is a reversible inhibitor of succinate dehydrogenase, 3-NP is an irreversible inhibitor.

Both 3-NP and malonate, when administered to rodents, have been shown to cause selective lesions in the striatum [145-148]. Supporting the hypothesis of an oxidative mechanism underlying 3-NP neuronal loss in the striatum is the observation that susceptibility to 3-NP neurotoxic effects are age-dependent [149].

3-NP is also an important model for HD neuropathology because 3-NP inhibition of succinate dehydrogenase is not limited to the striatum: like mutant huntingtin protein that is expressed systemically, intraperitoneal (i.p.) injection of 3-NP causes the preferential neurodegeneration of the striatum. Furthermore, the loss of neurons in cortical regions in advanced cases of HD is mimicked in the late stages of severe 3-NP toxicity. In addition, chronic 3-NP administration also follows the progression of hyperactivity to hypoactivity seen in HD [150]. Taken together, these observations suggest the mechanisms behind neuropathology in 3-NP toxicity may be similar to that of mutant huntingtin protein.

**Evidence of Oxidative Stress Caused by 3-NP**

The use of 3-NP as an animal model of HD has yielded evidence implicating oxidative stress. Apoptotic and excitotoxic mechanisms have been investigated in 3-NP toxicity [151]. Addition of 3-NP to neuronal cell cultures results in a dose-dependent increase in neuron death after 48 hours. During such studies, some neurons underwent rapid necrotic cell death while others exhibited a slow apoptotic cell death over the 48 hour time frame, suggesting both are mechanisms of 3-NP induced cell death. The rapid necrosis was inhibited by MK-801, an allosteric inhibitor of NMDA receptors; however, the delayed apoptosis was not, suggesting the former is excitotoxic while the later mechanism of cell death is not [151].

The excitotoxic mechanism of 3-NP neurodegeneration is consistent with an oxidative theory. Activation of NMDA receptors has been shown to lead to generation of superoxide radicals [152]. Other evidence has also linked excitotoxicity with free radical production [153]. 3-NP inhibition of the electron transport chain results in lower ATP levels, which in turn impairs ion pumps needed for the maintenance of neuronal membrane electrochemical polarity. The loss of membrane polarity leads to the loss of the voltage-dependent Mg$^{2+}$ block in the NMDA receptor ion channel. This causes ambient levels of glutamate to become excitotoxic [148,154]. The loss of the ion channel block also allows a dramatic increase in the flux of calcium into the cell which results in the activation of neuronal nitric oxide synthase. Calcium influx into the cell also further impairs mitochondrial function [152,155].

There is extensive indirect evidence of oxidative damage caused by 3-NP toxicity [156]. An increase in free fatty acid release has been associated with 3-NP neurotoxicity. The release of free fatty acids can be caused by the activity of phospholipase A$_2$, which cleaves fatty acid esters and is activated by lipid peroxidation [30,72,157]. Free fatty acid metabolism may itself contribute to the production of reactive oxygen species and increased oxidative stress [158]. Rodents injected with both 3-NP and salicylate also exhibit an increase in levels of 2,3-dihydroxybenzoic acid (DHBA) and 2,5-DHBA, both of which are metabolic products of salicylate in the presence of hydroxyl radicals [159]. Schulz et al. (1995) also reported evidence of peroxynitrite-mediated oxidative stress manifested by an increase in 3-nitrotyrosine. This suggests both hydroxyl radical and peroxynitrite are involved in 3-NP neurotoxicity.

Several factors which attenuate 3-NP neurotoxicity also suggest an underlying oxidative mechanism. Neuroprotection against 3-NP by caloric restriction [62,160] has been reported. Caloric restriction has been linked to a decrease in basal levels of oxidative damage, as noted above [56]. In addition, creatine and acetyl-L-carnitine have also been reported to offer neuroprotection against 3-NP toxicity [161,162].

Among the most compelling evidence for 3-NP-induced oxidative damage is an alteration in the levels of endogenous antioxidants observed in animals treated with 3-NP [163,164]. 3-NP induces an increase in catalase and
superoxide dismutase activities. Both of these enzymes reduce concentrations of reactive oxygen species (ROS), and an increase in their activation suggests an increase in ROS concentrations [163]. These researchers also observed a decrease in glutathione in response to 3-NP administration. Glutathione is a major endogenous antioxidant, and the decrease in glutathione levels implies the presence of free radicals [25,28]. Furthermore, 3-NP-induced neurotoxicity is accentuated in mice deficient in cellular glutathione peroxidase [165]. In contrast, vulnerability to 3-NP induced neurotoxicity is attenuated in mice overexpressing copper/zinc superoxide dismutase [164], suggesting that elevation in the endogenous antioxidant defense system may be protect against oxidative damage initiated by 3-NP toxicity. Consistent with this notion, oxidative stress conditions caused by 3-NP are also attenuated in mice overexpressing Bcl-2 [166]. The gene product of Bcl-2 has previously been shown to inhibit apoptosis [167], to inhibit necrotic neuronal cell death caused by glutathione depletion [168], to protect against peroxides in a dose-dependent manner [169,170], and protect cells against lipid peroxidation [32].

Direct evidence of oxidized proteins has also been found in HD model systems employing 3-NP. EPR, in conjunction with protein specific spin labels, has been used to observe protein conformational changes in synaptosomal membranes in both the striatum and the cortex of rats treated with 3-NP [36]. The W/S ratio of MAL-6-labeled striatal or cortical synaptosomal membranes was significantly lowered, consistent with oxidized proteins. In addition, direct observation of increased carbonyl functionality incorporated in synaptosomal membrane proteins in rats treated with 3-NP has been reported [36]. As noted above, protein carbonyls are an index of protein oxidation [5], and an increase in protein carbonyls is direct evidence that 3-NP neurotoxicity is associated with oxidative damage [36]. Furthermore, by observing protein oxidation at three days of 3-NP injection, when striatal lesions require four days of 3-NP injection i.p., this study reported evidence of oxidative stress prior to the observance of neuron loss in the striatum [36], consonant with the notion that the former may cause the latter.

Given the suggested role of oxidative stress in 3-NP toxicity, it is not surprising that exogenous antioxidants such as free radical spin traps have been investigated as agents to attenuate 3-NP neurodegeneration. The spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) has been reported to protect against 3-NP-induced striatal lesions [145]. The spin trap 5-dithioxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO), a more stable phosphorylated analogue of DMPO, has also been shown to protect against 3-NP-induced neurodegeneration in the striatum and, in addition, attenuate protein oxidation [37]. The commonly employed α-phenyl-tertiary-butynitrone (PBN) spin trap has been reported to exacerbate 3-NP toxicity, which has been attributed to this spin trap interfering with the pathways of 3-NP metabolic degradation [171,172]. Elevation of glutathione levels, via injection of the glutathione precursor N-acetyl-cysteine [28,173], has also been shown to protect against 3-NP-induced striatal neurodegeneration and protein oxidation [37], consistent with 3-NP induced oxidative stress.

Possible Sources of 3-NP-induced Oxidative Damage

Several sources of oxidative damage have been suggested to play a role in 3-NP neurotoxicity. As mentioned above, excitotoxicity, presumably caused by activation of NMDA receptors, may be one possible source. The NMDA receptor inhibitor MK-801 has been shown to protect against 3-NP-mediated neurotoxicity by some researchers [174-176]. Others, however, have failed to find evidence of NMDA receptor activation [177,178]. Our results show a limited, but significant, attenuation of 3-NP induced protein oxidation, suggesting NMDA receptor activation contributes to oxidative damage, but may not be the sole, or even principal, source of ROS [179]. Similar conclusions have been suggested by the results of others, who report NMDA receptor antagonists attenuate, but do not prevent neuronal death [174,180]. Part of this apparent discrepancy may be related to the known differences in 3-NP responses in different species and strains within the same species [181] used in these various studies.

Another possible source of oxidative damage in the 3-NP model is mitochondrial dysfunction. Any inhibition of mitochondrial energy production, such as that caused by the 3-NP inhibition of complex II, could conceivably cause an increase in oxygen flux through the mitochondria, leading to increased generation of ROS. Consistent with this notion, we showed that in-vivo 3-NP injection caused increased mitochondrial protein carbonyl levels in striatum and cortex isolated from rats [179]. It is possible that mitochondrial oxidation is caused via activation of NMDA receptors. Mitochondria can act as an intracellular storage compartment for calcium, and activation of NMDA receptors could lead to influx of calcium into the mitochondria, leading to mitochondrial oxidation. We showed, however, that MK-801 does not attenuate 3-NP-mediated mitochondrial protein oxidation, suggesting that mitochondrial oxidation may not be related to NMDA receptor activation by 3-NP [179].

3-NP-induced oxidative stress has also been suggested to result from an inflammatory response, for which evidence exists. For example, 3-NP-induced striatal lesions are infiltrated with neutrophils and are associated with immunoreactivity to serum/immune complement factors (C3b/C4b4) [182]. Furthermore, 3-NP neurotoxicity has been shown to lead to an increase in the levels of tumor necrosis factor-α [183], a pro-inflammatory cytokine, and increased expression of inducible nitric oxide synthase [184].

There is also evidence that dopamine metabolism may play a role in 3-NP neurotoxicity. Catechol chemistry involving dopamine has been shown to generate ROS when metabolized by the enzyme monoamine oxidase (MAO) to 3,4-dihydroxyphenylacetaldehyde (DOPAC). The conversion of dopamine to DOPAC causes the concomitant production of \( \text{H}_2\text{O}_2 \), from which hydroxyl radicals can be generated through Fenton chemistry. If dopamine metabolism is involved with 3-NP neurotoxicity, this would be of particular interest since striatal neurons are much more densely innervated with dopaminergic fibers than is the rest...
Fig. (2). Rats were injected with saline vehicle, 3-nitropropionic acid (20 mg/kg body mass, i.p.), or with 3-NP and the MAO inhibitors clorgyline and deprenyl (5 mg/kg body mass each, i.p.) for 4 days. 24 hours after final injections, animals were decapitated and the brains removed. Striatum and cortex were isolated and protein carbonyls were assayed with immunochemical staining. As previously reported, animals treated with 3-NP only exhibited significantly higher levels of protein carbonyls in both cortex and striatum when compared to control animals [36]. Co-treatment with MAO inhibitors significantly lowered protein carbonyl levels in the striatum when compared to animals treated with 3-NP only. MAO inhibitors did not, however, attenuate protein carbonyl levels in the cortex. N=6 for all groups.

of the brain. This might provide a possible mechanism for preferential striatal neuron loss in the 3-NP model of HD. In support of this hypothesis, the MAO-A and MAO-B inhibitors, deprenyl and clorgyline, respectively, have been reported to significantly reduce striatal lesions caused by malonate and 3-NP [185]. In addition, dopamine levels have been observed to be elevated in rodents treated with malonate [186]. As shown in Fig. (2), our results suggest that inhibition of MAO-A and MAO-B, using clorgyline and deprenyl, slightly attenuates protein oxidation in the striatum of rats treated with 3-NP, but not in the cortex. The modest level of protection, however, suggests that while dopamine metabolism may contribute to 3-NP-induced oxidative damage, it likely is not the only source of ROS.

Taken together, the results described above suggest that 3-NP may lead to the generation of ROS and subsequent oxidative damage via multiple pathways.

Huntingtin Overexpressing Transgenic Mice

The discovery of the genetic mutation which causes Huntington’s disease has allowed for the development of transgenic models of the disorder [187]. Though the use of the transgenic model is more recent, there is some support for the oxidative stress hypothesis. Mice expressing the mutant huntingtin have been shown to exhibit proteolytic cleavage of a GST-huntingtin fusion protein which leads to the formation of insoluble, high molecular weight protein aggregates reminiscent of beta-amyloid fibrils in Alzheimer’s disease or alpha-synuclein in Parkinson’s disease. Aggregated, soluble Aβ(1-42) has been linked to oxidative damage [14,94,108,188-190]. Mice with polyglutamate expansion regions less than the pathogenic range do not exhibit such aggregation. Other researchers have shown that caspase-1 inhibition attenuates progression of neuropathology in mice expressing huntingtin with an expanded glutamate region [191], suggesting an oxidative process. The most compelling evidences for an oxidative mechanism in the huntingtin transgenic model of HD are (a) the observation that striatal lipid peroxidation (measured by TBARS) parallels the expression of a neurological phenotype [192]; and (b) the observation of mitochondrial dysfunction [193]. These latter researchers report increased expression of inducible nitric oxide synthase and elevated levels of nitrotyrosine, suggesting peroxynitrite and nitric oxide may play a role in oxidative stress in these transgenic animals. These researchers also report a significant reduction in aconitase and mitochondrial complex IV activities in the striatum, suggesting mitochondrial dysfunction in these animals.

Similar to the case with animal models of AD discussed above, transgenic worms also have been used as models of HD. For example, studies of polyglutamine-mediated dysfunction and apoptotic death of ASH sensory neurons in young and old C.elegans showed that polyglutamine tracts of greater than 150 residues led to neurodegeneration and protein aggregation, reminiscent of HD [194,195]. Others showed that in C.elegans cleavage of huntingtin by apopain, a proapoptotic cysteine protease, was modulated by
polyglutamine tracts [196], from which the authors conclude that HD might be a disorder of associated with inappropriate apoptosis. Since apoptosis and protein aggregation have been associated with Aβ-mediated oxidative damage and neurotoxicity [15,197], further investigations of oxidative stress in these invertebrate models of HD should be pursued.

How aggregated huntingtin protein leads to oxidative stress and selective vulnerability of striatal spiny neurons in HD brain remains unknown. One hypothesis is that a "gain of function" occurs. For example, the expanded polyglutamine region in HD could force the normal huntingtin into a more β-sheet conformation that could affect its interaction with other proteins. Such interactions could alter normal metabolic processing in mitochondria or could alter expression of mitochondrial-resident proteins, components of which are produced by nuclear DNA. Other scenarios are possible.

Although, clearly, additional studies into oxidative stress associated with HD and animal models of this age-related neurodegenerative disorder are necessary, the similarities of the pathologies and behavior in the 3-NP and huntingtin transgenic models of HD, coupled with the oxidative stress evident in these models of HD, suggest strongly that this autosomal dominant disease is associated with oxidative stress. Studies to test this hypothesis explicitly are underway in our laboratory.

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REFERENCES

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